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(71) Applicant *(for all designated States except US)*: **THE GENERAL HOSPITAL CORPORATION** [US/US]; Fruit Street, Boston, Massachusetts 02114 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **GARDELLA, Thomas, J.** [US/US]; 1045 Greendale Avenue, Needham, Massachusetts 02492 (US).

(74) Agents: BUGAISKY, Lawrence, B. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., 1100 New York Avenue, N.W., Washington, DC 20005 (US).

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(54) Title: CONFORMATIONALLY CONSTRAINED PARATHYROID HORMONE (PTH) ANALOGS

(57) Abstract: The invention provides novel P1R polypeptide antagonists. These antagonists contain amino acid substitutions at selected positions in truncated PTH and PRHrP polypeptides and function by binding selectively to the juxtamembrane ("J") domain of the receptor. The J domain is the region of the receptor that spans the seven transmembrane domain and the extracellular loops.

CONFORMATIONALLY CONSTRAINED PARATHYROID HORMONE (PTH) ANALOGS

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH AND DEVELOPMENT

[0001] Statement under MPEP 310. The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. DK-11794 awarded by the National Institutes of Health.

[0002] Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] The present invention relates to conformationally constrained parathyroid hormone (PTH) and parathyroid hormone related protein (PTHrP) analogs, and methods of preparing and using these analogs.

Background Art

Parathyroid hormone

[0004] Parathyroid hormone (PTH), an 84 amino acid peptide, is the principal regulator of ionized blood calcium in the human body (Kronenberg, H.M., *et al.*, In *Handbook of Experimental Pharmacology*, Mundy, G.R., and Martin, T.J., (eds), pp. 185-201, Springer-Verlag, Heidelberg (1993)). Regulation of calcium concentration is necessary for the normal function of the gastrointestinal, skeletal, neurologic, neuromuscular, and cardiovascular systems. PTH synthesis and release are controlled principally by the serum calcium level; a low level

stimulates and a high level suppresses both hormone synthesis and release. PTH, in turn, maintains the serum calcium level by directly or indirectly promoting calcium entry into the blood at three sites of calcium exchange: gut, bone, and kidney. PTH contributes to net gastrointestinal absorption of calcium by favoring the renal synthesis of the active form of vitamin D. PTH promotes calcium resorption from bone indirectly by stimulating differentiation of the bone-resorbing cells, osteoclasts. It also mediates at least three main effects on the kidney: stimulation of tubular calcium reabsorption, enhancement of phosphate clearance, and promotion of an increase in the enzyme that completes synthesis of the active form of vitamin D. PTH is thought to exert these effects primarily through receptor-mediated activation of adenylate cyclase and/or phospholipase C.

[0005] Disruption of calcium homeostasis may produce many clinical disorders (*e.g.*, severe bone disease, anemia, renal impairment, ulcers, myopathy, and neuropathy) and usually results from conditions that produce an alteration in the level of parathyroid hormone. Hypercalcemia is a condition that is characterized by an elevation in the serum calcium level. It is often associated with primary hyperparathyroidism in which an excess of PTH production occurs as a result of a parathyroid gland lesion (*e.g.*, adenoma, hyperplasia, or carcinoma). Another type of hypercalcema, humoral hypercalcemia of malignancy (HHM), is the most common paraneoplastic syndrome. It appears to result in most instances from the production by tumors (*e.g.*, squamous, renal, ovarian, or bladder carcinomas) of a class of protein hormone which shares amino acid homology with PTH. These PTH-related proteins (PTHrP) appear to mimic certain of the renal and skeletal actions of PTH and are believed to interact with the PTH receptor in these tissues.

PTH Derivatives

[0006] PTH derivatives include polypeptides that have amino acid substitutions or are truncated relative to the full length molecule. A 14, a 21 and a 34 amino acid amino-terminal truncated form of PTH, as well as a C-terminal truncated form have been studied. Additionally, amino acid substitutions within the truncated polypeptides have also been investigated.

[0007] Synthetic PTH(1-34) exhibits full bioactivity in most cell-based assay systems, has potent anabolic effects on bone mass in animals and has recently been shown to reduce the risk of bone fracture in postmenopausal osteoporotic women (Neer, R.M., *et al., N.E.J.M. 344*:1434-1441 (2001); Dempster, D.W., *et al., Endocr Rev 14*:690-709 (1993)). PTH acts on the PTH/PTHrP receptor (P1R), a class II G protein-coupled heptahelical receptor that couples to the adenylyl cyclase/CAMP and phospolipase C/inositol phosphate (IP) signaling pathway (Rippner, H., *et al., Science 254*:1024-1026 (1991)). Deletion analysis studies have shown that the amino-terminal residues of PTH play a crucial role in stimulating the P1R to activate the cAMP and IP signaling pathways (Tregear, G.W., *et al., Endocrinology 93*:1349-1353 (1973); Takasu, H., *et al., Biochemistry 38*:13453-13460 (1999)). Crosslinking and receptor mutagenesis studies have indicated that residues in the amino-terminal portion of PTH interact with the extracellular loops and extracellular ends of the seven transmembrane helices, which reside within the juxtamembrane region of the receptor (Bergwitz, C., *et al., J. Biol. Chem. 271*:26469-26472 (1996); Hoare, S.R.J., *et al., J. Biol. Chem 276*:7741-7753 (2001); Behar, V., *et al., J. Biol. Chem. 275*:9-17 (1999); Shimizu, M., *et al., J. Biol. Chem. 275*:19456-19460 (2000); Luck, M.D., *et al., Molecular Endocrinology 13*:670-680 (1999)).

[0008] Most current P1R antagonists are N-terminally truncated analogs of PTH(1-34) or PTHrP (1-36) (e.g. PTHrP(5-36)). These antagonists recognize the receptor's amino-terminal extracellular ("N") domain with high binding affinity.

However, the N-terminal truncation results in the inability of the PTH or PTHrP peptide to signal through the receptor, thereby acting as an antagonist.

α-Helix Stabilizers

[0009] The first 34 amino acids of PTH and PTHrP contain sufficient information for high affinity P1R binding and potent induction of P1R-mediated signaling responses (Neer, RM, *et al.*, *N.E.J.M. 344*: 1434-1441(2001)). Short N-terminal fragments of PTH, such as PTH(1-14) and PTH(1-11) exhibit extremely weak binding affinities (Kd>>100μM) but are nonetheless capable of eliciting cAMP-signaling responses, albeit with potencies (EC50s≥100μM) that are substantially weaker than that of PTH(1-34)(EC50~2nM)(Luck, *MD et al.*, *Molecular Endocrinology 13*: 670-680(1999)). Recently, it has been discovered that a series of modified PTH(1-14) and PTH(1-11) analogs exhibit signaling potencies that are nearly, or even fully, equal to that of PTH(1-34)(Shimizu, M *et al., Endocrinology 142*: 3068-3074(2001); Shimizu, M. *et al., J. Biol. Chem. 276*: 490003-49012(2001); Shimizu, M. *et al., J.Biol.Chem. 275*: 21836-21843(2000)). One such type of a modifier is a lactam bridge, which is a side chain-to-side chain amide bridge formed between a basic lysine residue and an acidic aspartame or glutamate residue (Condon, SM. *et al., J. Am. Chem. Soc. 122*: 3007-3014 (2000)). Lactam bridge formation is a well-known method by which the bioactive conformation of peptides may be deduced (*See* Id.). Incorporation of lactam bridges between residues 13 and 17; 18 and 22; and 26 and 30 in human PTH (1-31) and (1-34) (hPTH) has shown bioactivity while retaining a helical conformation (*see* Id.). Additionally, these modifications of hPTH(1-31) and hPTH(1-34) suggest that an α-helix may be the preferred bioactive conformation for the N-terminal portion of PTH (Shimizu, N. *et al., J. Biol. Chem. 276*: 490003-49012(2001)).

[0010] Recently, it was also discovered that PTH(1-14) analogs containing the α,α-disubstitued amino acid, α-amino-isobutyric acid (Aib) at positions 1 and/or

3, have 10- to 100- fold higher affinities and cAMP signaling potencies than do their counterpart peptides containing alanine at these positions (Shimizu, N. *et al.* *J. Biol. Chem. 276*: 49003-49012 (2001)).

BRIEF SUMMARY OF THE INVENTION

[0011] The invention provides novel P1R polypeptide antagonists. These antagonists contain amino acid substitutions at selected positions in truncated PTH and PRHrP polypeptides and bind selectively to the juxtamembrane ("J") domain of the receptor. The J domain is the region of the receptor (P1R) containing the seven transmembrane helices and extracellular loops. N-terminal PTH antagonist analogs of the present invention, that bind to the J domain of the P1R, would be useful for treating conditions relating to PTH/P1R hyperactivity (*e.g.,* primary hyperparathyroidism, Jansen's chondrodysplasia). In addition, these analogs would be useful for identifying other ligands (*e.g.,* utilizing a high-throughput screen) that bind to P1R, such as small molecule PTH mimetic compounds. Moreover, these analogs could be used for pharmacologically analyzing P1R ligands for their selectivity, for example for the J domain.

[0012] The invention provides derivatives of PTH (1-21), PTH(1-20), PTH(1-19), PTH(1-18), PTH(1-17), PTH(1-16), PTH(1-15), PTH(1-14), PTH(1-13), PTH(1-12), PTH(1-11) and PTH(1-10) polypeptides, wherein said derivatives bind selectively to the J domain of P1R and act as antagonists or inverse agonists of P1R activity. The invention also provides methods of making such peptides. Further, the invention encompasses compositions and methods for use of such peptides in receptor-ligand assays. Additionally, the invention provides compositions and methods for use of such peptides in treating conditions associated with elevated levels of PTH, including, for example, hypercalcemia, and conditions related to hyperparathyroidism.

[0013] In one aspect, the invention is directed to a peptide selected from a group consisting of: $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}His$X_{05}X_{06}X_{07}$LysX_{08} (SEQ ID NO: 1),

wherein X_{01} and X_{03} are α-helix stabilizing residues (*e.g.*, Ac_5c, Ac_3c, Deg, Aib or the desamino form of Ac_5c, Ac_3c, Deg, or Aib); X_{02} is Trp, Bpa, Arg or Val; X_{04} is Met or Nle; X_{05} is Gln, Deg or Asn; X_{06} is Har or Leu; X_{07} is an α-helix stabilizing residue (*e.g.*, Aib), Ala or Gly; and X_{08} is an α-helix stabilizing residue (*e.g.*, Aib), Trp, Tyr or His; fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13; pharmaceutically acceptable salts thereof; and N- or C-derivatives thereof. In one embodiment of the present invention, the peptide above consists essentially of $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}His$X_{05}X_{06}X_{07}$LysX_{08} (SEQ ID NO: 1), wherein X_{01} and X_{03} are α-helix stabilizing residues (*e.g.*, Ac_5c, Ac_3c, Deg, Aib or the desamino form of Ac_5c, Ac_3c, Deg, or Aib); X_{02} is Trp, Bpa, Arg or Val; X_{04} is Met or Nle; X_{05} is Gln, Deg or Asn; X_{06} is Har or Leu; X_{07} is an α-helix stabilizing residue (*e.g.*, Aib), Ala or Gly; and X_{08} is an α-helix stabilizing residue (*e.g.*, Aib), Trp, Tyr or His; fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13; pharmaceutically acceptable salts thereof; and N- or C- derivatives thereof.

[0014] The invention is further drawn to fragments of the peptide of SEQ ID NO: 1, in particular $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}His$X_{05}X_{06}X_{07}$Lys (SEQ ID NO: 2), $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}His$X_{05}X_{06}X_{07}$ (SEQ ID NO: 3), $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}His$X_{05}X_{06}$ (SEQ ID NO: 4) and $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}HisX_{05} (SEQ ID NO: 5). The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides. An embodiment of the invention is drawn to any of the above recited polypeptides, wherein said polypeptide contains a C-terminal amide.

[0015] Another aspect of the invention is directed to a peptide consisting of X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}LeuAlaSerValX_{08}ArgX_{09} (SEQ ID NO: 6), wherein X_{01} and X_{02} are α-helix stabilizing residues (*e.g.*, Ac_5c,Ac_3c, Deg, or Aib), X_{03} is Aib, Gln, Deg or Asn, X_{04} is Met or Nle, X_{05} is Har or Leu, X_{06} is an α-helix stabilizing residue (e.g. Aib), Ala or Gly, X_{08} is an α-helix stabilizing residue (e.g. Aib) or Lys, and X_{07} is an α-helix stabilizing residue (e.g.

Aib), Trp or His, X_{08} is Arg or Glu and X_{09} is Tyr or Met; wherein said peptide binds selectively to the J domain of P1R.

[0016] The invention is further drawn to fragments of the peptide of SEQ ID NO: 6, in particular X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}LeuAlaSerValX_{08}Arg (SEQ ID NO: 7), X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}LeuAlaSerValX_{08} (SEQ ID NO: 8),X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}LeuAlaSerVal (SEQ ID NO:9), X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}LeuAlaSer (SEQ ID NO: 10), X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}LeuAla (SEQ ID NO: 11) and X_{01}BpaX_{02}GluIleGlnLeuX_{03}His$X_{04}X_{05}X_{06}$LysX_{07}Leu (SEQ ID NO:12). The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides. An embodiment of the invention is drawn to any of the above recited polypeptides, wherein said polypeptide contains a C-terminal amide.

[0017] The invention is further drawn to any of the above polypeptides labeled with a label selected from the group consisting of: a radiolabel, a flourescent label, a bioluminescent label, or a chemiluminescent label. In an embodiment the radiolabel is ^{125}I or ^{99m}Tc.

[0018] Embodiments of the peptide according to the present invention include: Ac5cBpaAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO:13), Ac_5cValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 14), (desNH2)Ac_5cValAibGluIleGlnLeuMetHisGlnHarAla-LysTrpNH_2(SEQ ID NO: 15), (desNH2)AibValAibGluIleGlnLeuMetHisGlnHarAla-LysTrpNH_2(SEQ ID NO: 16), Ac_5cTrpAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 17), Ac_5cBpaAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 18), Ac_5cArgAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 19), DegValDegGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 20), DegTrpDegGluIleGlnLeuMetHisGlnHarAlaLysTrpNH2 (SEQ ID NO: 21), DegBpaDegGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 22),

$Ac_5cTrpAibGluIleGlnLeuNleHisGlnHarAlaLysTyrNH_2$ (SEQ ID NO: 23), $Ac_5cBpaAibGluIleGlnLeuNleHisGlnHarAlaLysTyrNH_2$ (SEQ ID NO: 24) and $DegBpaDegGluIleGlnLeuNleHisGlnHarAlaLysTrpLeuAlaSerValArgArgTyrNH_2$ (SEQ ID NO: 25).

[0019] In another aspect, the invention is directed to methods of making any of the above peptides, including a method wherein the peptide is synthesized by solid phase synthesis. The invention is also directed to a method of making any of the above peptides, wherein the peptide is protected by FMOC.

[0020] In a further aspect of the invention, this invention also provides pharmaceutical compositions comprising a peptide of the present invention and a pharmaceutically acceptable excipient and/or a pharmaceutically acceptable solution such as saline or a physiologically buffered solution.

[0021] This invention also provides a method for treating mammalian conditions characterized by increased PTH activity, such as, for example hypercalcemia, which method comprises administering to a subject in need thereof an effective amount of a peptide of the present invention. An embodiment of the invention is drawn to conditions such as hypercalcemia. Additional embodiments include using an effective amounts of the polypeptide of about 0.01 μg/kg/day to about 1.0 μg/kg/day wherein the polypeptide may be administer parenterally, subcutaneously or by nasal insufflation.

[0022] In accordance with yet a further aspect of the invention, this invention also provides a method for using the J-domain selective peptides in ligand-receptor assays. According to the method, the peptide may be labeled with a label selected from the group consisting of: radiolabel, flourescent label, bioluminescent label, or chemiluminescent label. Examples of a suitable radiolabel are ^{125}I or ^{99m}Tc.

[0023] The invention is further related to a method of blocking increases in cAMP in a mammalian cell having PTH-1 receptors, said method comprising contacting the cell with a sufficient amount of the polypeptide of the invention to block increases in cAMP.

BRIEF DESCRIPTION OF THE FIGURES

[0024] FIG. 1. PTH Analogs Utilized and Their Amino Acid Sequences. All peptides are derived from the rat PTH sequence. Non-conventional amino acids include homoarginine (Har), norleucine (Nle); 1-aminoisobutyric acid (Aib); 1-aminocyclopropane-1-carboxylic acid (Ac_3c), diethylglycine (Deg), 1-aminocyclopentane-1-carboxylic acid (Ac_5c). The amino acids conferring antagonist properties to the peptides are in boldface type. The asterisk indicates the iodinated tyrosine.

[0025] FIG. 2. Binding of PTH Analogs in HKRK-B28 Cells. The parent peptide was [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 and derivatives thereof were substituted at positions 1, 2 and/or 3, as indicated. Binding assays were performed with ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]PTH(1-15)NH_2. Data are from the number of experiments indicated (n), each performed in duplicate.

[0026] FIG. 3. Functional Responses in HKRK-B28 Cells. Binding (A and B) and cAMP agonism/partial agonism assays (C) were performed in HKRK-B28 cells. The parent peptide was [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 and derivatives thereof were substituted at positions 1, 2 and/or 3, as indicated. Binding assays (4h at 15°C) were performed with ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]PTH(1-15)NH_2 tracer. cAMP assays were performed at RT for 30 min. Relative to the parent, the substituted analogs displayed reduced agonist activity.

[0027] FIG. 4. cAMP Responses in HKRK-B28 Cells. The parent peptide, [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2, and derivatives thereof substituted at positions 1, 2 and/or 3, as indicated, were assayed for cAMP agonist responses in HKRK-B28 cells. The parent peptide functions as a fully potent and efficacious agonist, the $Deg^{1,3}$-substituted analog is a partial agonist, and the Bpa^2-substituted analogs lack agonist activity.

[0028] FIG. 5. Antagonism Assays in HK-RK-B28 Cells. cAMP antagonism

assays were performed in HKRK-B28 cells. Cells were treated with the J domain-selective agonist, $[Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1\text{-}14)NH_2$ (parent) at 10 nM, either alone (none) or with a candidate antagonist peptide (10 μM), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated, or $[I^5,W^{23},Y^{36}]PTHrP(5\text{-}36)$ analog. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

[0029] FIG. 6. Antagonism Assays in COS-7 Cells. cAMP antagonism assays were performed in COS-7 cells transfect with the wild-type P1R (A), or a constitutively active P1R derivative having the first 9 residues of PTH tethered to TM1 of the P1R and in place of the P1R N-terminal domain (inset), B). In A, cells were treated with the J domain-selective agonist, $[Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1\text{-}14)NH_2$ (parent) at 1 nM, alone (none) or with a candidate antagonist peptide (10 μM), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated, or $[I^5,W^{23},Y^{36}]PTHrP(5\text{-}36)$ analog. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

[0030] FIG. 7. Inverse Agonist Responses in COS-7 Cells. COS-7 cells were transfected with the constitutively active P1Rs: P1R-H223R (A), P1R-T410P (B), P1R-H223R/T410P (C), or P1R-I458R (D) and then were incubated (30 min at RT) either in the absence of peptide (none) or in the presence of the indicated antagonist/inverse agonist peptide (10 μM), and cAMP was measured by RIA. Asterisks indicate significant reductions in cAMP levels, compared to untreated cells (none).

[0031] FIG. 8. "N" versus "J" Domain selectivity of PIR Antagonists in COS-7 Cells. cAMP antagonism assays were performed in COS-7 cells transfect with the wild-type P1R (A), or a P1R derivative (P1R-delNt) having most (residues 24-181) of the P1R N domain deleted (B). Cells were treated with the agonist $[Aib^{1,3},Tyr^{34}]hPTH(1\text{-}34)NH_2$ ($[Aib^{1,3}]PTH(1\text{-}34)$), which utilizes both N and J domains for affinity/potency, or with $[Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1\text{-}$

14)NH_2 ([$Ac5c^1$]PTH(1-14)), which uses only the J domain for affinity/potency, at the concentrations indicated in the key, so as to elicit half-maximum cAMP responses in the absence of antagonist (none). The analogs PTHrP(5-36) and $Deg^{1,3}$,Bpa^2-PTH(1-21) were added at 1x10-5 M, as indicated. On the WT receptor, PTHrP(5-36) antagonizes PTH(1-34) analog more effectively than does $Deg^{1,3}$,Bpa^2-PTH(1-21), but the PTH(1-21) analog antagonizes PTH(1-14), more effectively than does PTHrP(5-36). On P1R-delNt, $Deg^{1,3}$,Bpa^2-PTH(1-21) antagonizes either agonist, whereas PTHrP(5-36) lacks antagonist capability. Thus, PTHrP(5-36) is an N domain-selective antagonist, whereas $Deg^{1,3}$,Bpa^2-PTH(1-21) is a J domain-selective antagonist. The analog $Deg^{1,3}$,Bpa^2-PTH(1-14) behaved similarly in these assays to $Deg^{1,3}$,Bpa^2-PTH(1-21).

[0032] FIG. 9. Competition Binding Assays in HKRK-B7 Cells. Binding assays were performed in HKRK-B7 cells, which express the wild-type hP1R, using ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]PTH(1-15)NH_2 as a tracer radioligand and the indicated unlabeled peptides as competitors. PTH(1-34) is [Tyr^{34}]hPTH(1-34)NH_2.

[0033] FIG 10. Competitive Antagonism on P1R-delNt. COS-7 cells transfected with P1R-delNt were stimulated with varying concentrations of the agonist [$Aib^{1,3}$,Tyr^{34}]hPTH(1-34)NH_2 ([$Aib^{1,3}$]PTH(1-34)), either in the absence of antagonist or in the presence of an antagonist, [$Deg^{1,3}$,Bpa_2,M]PTH(1-14) or [$Deg^{1,3}$,Bpa^2,M]PTH(1-21) each at 1x10-5 M, as indicated in the figure key. Each antagonist causes a parallel, right-ward shift in the agonist dose-response curve , which is consistent with a competitive mechanism of inhibition.

[0034] FIG 11. A model of the interaction between PTH derivatives of the present invention and the J domain of the P1R receptor.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0035] "A," "an," "the," and the like, unless otherwise indicated, may include plural forms. Also, reference to singular forms embrace plural forms. Therefore, for example, peptide also encompasses peptides.

[0036] *Amino Acid Sequences*: The amino acid sequences in this application use either the single letter or three letter designations for the amino acids. These designations are well known to one of skill in the art and can be found in numerous readily available references, such as for example in *Cooper, G.M., The Cell* 1997, ASM Press, Washington, D.C. or Ausubel et al., *Current Protocols in Molecular Biology,* 1994 . Where substitutions in a sequence are referred to, for example, as Ser-3 -->Ala or [Ala^3]peptide, this means that the serine in the third position from the N-terminal end of the polypeptide is replaced with another amino acid, Alanine in this instance.

[0037] In the present application, "Aib" refers to α-aminoisobutyric acid; "Har" refers to homoarginine; "Nle" refers to norleucine; AC_3C refers to 1-aminocyclopropane-1-carboxylic acid; AC_5C refers to 1-aminocyclopentane-1-carboxylic acid; Har refers to homoarginine; Deg refers to diethylglycine; and other amino acids are in either the conventional one- or three-letter codes.

[0038] *Biological Activity of the Protein:* This expression refers to any biological activity of the polypeptide. Examples of these activities include, but are not limited to metabolic or physiologic function of compounds of SEQ ID NO: 1 or derivatives thereof, including similar activities or improved activities, or those activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of the above-described compounds.

[0039] *Derivative or Functional Derivative:* The term "derivative" or "functional derivative" is intended to include "variants," the "derivatives," or "chemical derivatives" of PTH molecules. A "variant" of a molecule such as for example,

a compound of SEQ ID NO: 1 or derivative thereof is meant to refer to a molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule such as for example, a compound of SEQ ID NO: 1 or derivative thereof is meant to refer to a non-natural molecule substantially similar to either the SEQ ID NO: 1 molecules or fragments thereof.

[0040] PTH derivatives contain changes in the polypeptide relative to the native PTH polypeptide of the same size. The sequence of the native PTH(1-14) polypeptide is the first fourteen amino acids of SEQ. ID NO: 17 (human PTH (1-21)) or SEQ. ID NO: 18 (rat PTH (1-21)). A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, two molecules that possess a similar activity, may be considered variants, derivatives, or analogs as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. PTH derivatives, however, need not have substantially similar biological activity to the native molecule. In some instances PTH derivatives may have substantially different activity than the native PTH. For example, a derivative may be either an antagonist or an agonist of the PTH receptor.

[0041] As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Examples of moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980) and will be apparent to those of ordinary skill in the art.

[0042] *Fragment:* A "fragment" of a molecule such as, for example, SEQ ID NO: 1 or derivative thereof is meant to refer to any polypeptide subset of these molecules.

[0043] *Fusion protein:* By the term "fusion protein" is intended a fused protein comprising compounds such as for example, SEQ ID NO: 1 or derivatives thereof, either with or without a "selective cleavage site" linked at its N-terminus, which is in turn linked to an additional amino acid leader polypeptide sequence.

[0044] *J domain:* The J domain is the domain of the P1R spanning the region of the receptor comprising the seven transmembrane domains and the extracellular loops.

[0045] *Polypeptide:* Polypeptide and peptide are used interchangeably . The term polypeptide refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids and include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in the research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.

[0046] Polypeptides may be branched and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translational modifications or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-

links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. *See*, for instance, *Proteins-Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Methods in Enzymol. 182*:626-646 (1990) and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci 663*:48-62 (1992).

PTH Analogs - Structural and Functional Properties

[0047] α-aminoisobutyric acid (Aib) was introduced into short N-terminal PTH peptide analogs. The numerous NMR studies of PTH(1-34) analogs, performed in a variety of polar or non-polar solvents, have generally indicated two domains of secondary structure: a stable C-terminal helix extending approximately from Ser-17 to Val-31, and a shorter and less stable amino-terminal helix, extending variably from Ser-3 to Lys-13, the two domain being connected by a bend or turn region (Marx, U.C., *et al., Biochem. Biophys. Res. Commun. 267*:213-220 (2000); Chen, Z., *et al., Biochemistry 39*:12766-12777 (2000); Marx, U.C., *et al., J. Biol. Chem. 270*:15194-15202 (1995); Marx, U.C., *et al., J. Biol. Chem. 273*:4308-4316 (1998); Pellegrini, M., *et al., Biochemistry 37*:12737-12743 (1998); Gronwald, W., *et al., Biol. Chem. Hoppe Seyler 377*:175-186 (1996); Barden, J.A., and Kemp, B.E., *Biochemistry 32*:7126-7132 (1993)). The recent crystallographic study of PTH(1-34) indicated a continuous α-helix extending from Ser-3 to His-32 and containing only a slight 15° bend at the midsection.

However, NMR data indicates that the N-terminal α-helix is relatively weak. Helix-stabilizing modifications, such as the introduction of Aib residues, offer significant benefits in terms of binding affinity to the P1R receptor, and result in short peptides (≤14 amino acids) with binding affinity that is comparable to PTH(1-34).

[0048] Described herein are novel "minimized" variants of PTH or PTHrP that are small enough to be deliverable by simple non-injection methods and that act as antagonists or inverse agonists by binding to the J domain of the P1R. The variants of the present invention contain substitutions in the first 21 amino acids of the polypeptide. The new polypeptides correspond to the 1-21, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 1-14, 1-13, 1-12, 1-11, and 1-10 amino acid sequence of the mature PTH polypeptide. The shorter variants (≤ PTH1-14) have a molecular weight of less than 2,000 daltons.

[0049] The primary amino acid sequence of the native human PTH(1-21) peptide (N-terminus to C-terminus) is SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAsnSerMetGluArgVal (SEQ ID NO: 26), whereas the primary sequence of the native rat PTH (1-21) is AlaValSerGluIleGlnLeuMetHisAsnLeuGlyLysHisLeuAlaSerValGluArgMet (SEQ ID NO. 27).

[0050] As protein products, compounds described herein are amenable to production by the techniques of solution- or solid-phase peptide synthesis and by in-situ synthesis using combination chemistry. The solid phase peptide synthesis technique, in particular, has been successfully applied in the production of human PTH and can be used for the production of these compounds (for guidance, *see* Kimura *et al., supra*, and see Fairwell *et al., Biochem. 22*:2691 (1983)). Success with producing human PTH on a relatively large scale has been reported by Goud *et al., in J. Bone Min. Res. 6(8)*:781 (1991). The synthetic peptide synthesis approach generally entails the use of automated synthesizers and appropriate resin as solid phase, to which is attached the C-terminal amino acid of the desired compounds of SEQ ID NO: 1 or derivatives thereof. Extension of the peptide in

the N-terminal direction is then achieved by successively coupling a suitably protected form of the next desired amino acid, using either FMOC- or BOC-based chemical protocols typically, until synthesis is complete. Protecting groups are then cleaved from the peptide, usually simultaneously with cleavage of peptide from the resin, and the peptide is then isolated and purified using conventional techniques, such as by reversed phase HPLC using acetonitrile as solvent and trifluoroacetic acid as ion-pairing agent. Such procedures are generally described in numerous publications and reference may be made, for example, to Stewart and Young, "Solid Phase Peptide Synthesis," 2nd Edition, Pierce Chemical Company, Rockford, IL (1984). It will be appreciated that the peptide synthesis approach is required for production of such as for example, SEQ ID NO: 1 and derivatives thereof which incorporate amino acids that are not genetically encoded, such as Aib.

[0051] In accordance with another aspect of the present invention, substituents may be attached to the free amine of the N-terminal amino acid of compounds of the present invention standard methods known in the art. For example, alkyl groups, e.g., C_{1-12} alkyl, may be attached using reductive alkylation. Hydroxyalkyl groups, e.g. C_{1-12} hydroxyalkyl, may also be attached using reductive alkylation wherein the free hydroxy group is protected with a t-butyl ester. Acyl groups, e.g., COE_1, may be attached by coupling the free acid, e.g., E_1COOH, to the free amino of the *N*-terminal amino acid. Additionally, possible chemical modifications of the C-terminal end of the polypeptide are encompassed within the scope of the invention. These modifications may modify binding affinity to the receptor.

[0052] Also contemplated within the scope of this invention are those compounds such as for example, SEQ ID NO:1 and derivatives thereof with altered secondary or tertiary structure, and/or altered stability, which retain selectivity for the J-domain of P1R and also retain antagonistic or reverse-agonist activity. Such derivatives might be achieved through lactam cyclization, disulfide bonds, or other means known to a person of ordinary skill in the art.

Utility and Administration of Compounds of the Invention

[0053] Compounds of the invention or derivatives thereof have multiple uses due, in part to their ability to act as antagonists or inverse agonists of P1R. The multiple uses of the peptides of the present invention include, *inter alia,* prevention and treatment of a variety of mammalian conditions manifested by increased activity and/or production of PTH or PTHrP, diagnostic probes, antigens to prepare antibodies for use as diagnostic probes and even as molecular weight markers. Being able to specifically substitute one or more amino acids in the PTH polypeptide permits construction of specific molecular weight polypeptides.

[0054] In particular, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of hypercalcemia and for treatment of hyperparathyroidism, Jansen's chondrodysplasia, or condition related thereto.

[0055] In certain embodiments, compounds of the present invention, or salts thereof, are administered in amounts necessary to treat a patient in need of treatment for conditions requiring antagonists of PTH receptors. In some embodiments, the compounds are administered between about 0.01 and 1 μg/kg body weight per day, preferably from about 0.07 to about 0.2 μg/kg body weight per day. For a 50 kg human female subject, the daily dose of biologically active compound is from about 0.5 to about 50 μgs, preferably from about 3.5 to about 10 μgs. In other mammals, such as horses, dogs, and cattle, higher doses may be required. This dosage may be delivered in a conventional pharmaceutical composition by a single administration, by multiple applications, or via controlled release, as needed to achieve the most effective results, preferably one or more times daily by injection. For example, this dosage may be delivered in a conventional pharmaceutical composition by nasal insufflation.

[0056] The selection of the exact dose and composition and the most appropriate delivery regimen will be influenced by, *inter alia*, the pharmacological properties

of the selected compounds of the invention, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient.

[0057] Representative delivery regimens include, without limitation, oral, parenteral, subcutaneous, transcutaneous, intramuscular and intravenous, rectal, buccal (including sublingual), transdermal, and intranasal insufflation.

[0058] Pharmaceutically acceptable salts retain the desired biological activity of the compounds of the invention without toxic side effects. Examples of such salts are (a) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; and salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalene disulfonic acids, polygalacturonic acid and the like; (b) base addition salts formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; or with an organic cation formed from N,N′-dibenzylethylenediamine or ethylenediamine; or (c) combinations of (a) and (b), e.g., a zinc tannate salt and the like. Pharmaceutically acceptable buffers include but are not limited to saline or phosphate buffered saline. Also included in these solutions may be acceptable preservative known to those of skill in the art. Like PTH, the PTH variants may be administered in combination with other agents useful in treating a given clinical condition.

[0059] A further aspect of the present invention relates to pharmaceutical compositions comprising as an active ingredient compounds of the invention or derivatives thereof of the present invention, or pharmaceutically acceptable salt thereof, in admixture with a pharmaceutically acceptable, non-toxic carrier. As mentioned above, such compositions may be prepared for parenteral (subcutaneous, transcutaneous, intramuscular or intravenous) administration,

particularly in the form of liquid solutions or suspensions; for oral or buccal administration, particularly in the form of tablets or capsules; for rectal, transdermal administration; and for intranasal administration, particularly in the form of powders, nasal drops or aerosols.

[0060] The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., (1985), incorporated herein by reference. Formulations for parenteral administration may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. For oral administration, the formulation can be enhanced by the addition of bile salts or acylcarnitines. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered spray. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

[0061] When formulated for nasal administration, the absorption across the nasal mucous membrane may be enhanced by surfactant acids, such as for example, glycocholic acid, cholic acid, taurocholic acid, ethocholic acid, deoxycholic acid, chenodeoxycholic acid, dehydrocholic acid, glycodeoxycholic acid, cyclodextrins and the like in an amount in the range between about 0.2 and 15 weight percent, preferably between about 0.5 and 4 weight percent, most preferably about 2 weight percent.

[0062] Delivery of the compounds of the present invention to the subject over prolonged periods of time, for example, for periods of one week to one year, may be accomplished by a single administration of a controlled release system containing sufficient active ingredient for the desired release period. Various controlled release systems, such as monolithic or reservoir-type microcapsules, depot implants, osmotic pumps, vesicles, micelles, liposomes, transdermal

patches, iontophoretic devices and alternative injectable dosage forms may be utilized for this purpose. Localization at the site to which delivery of the active ingredient is desired is an additional feature of some controlled release devices, which may prove beneficial in the treatment of certain disorders.

[0063] One form of controlled release formulation contains the polypeptide or its salt dispersed or encapsulated in a slowly degrading, non-toxic, non-antigenic polymer such as copoly(lactic/glycolic) acid, as described in the pioneering work of Kent, Lewis, Sanders, and Tice, U.S. Pat. No. 4,675,189. The compounds or their relatively insoluble salts, may also be formulated in cholesterol or other lipid matrix pellets, or silastomer matrix implants. Additional slow release, depot implant or injectable formulations will be apparent to the skilled artisan. See, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson ed., Marcel Dekker, Inc., New York, 1978, and R. W. Baker, Controlled Release of Biologically Active Agents, John Wiley & Sons, New York, 1987.

PTH Analog Receptor-Signaling Activities

[0064] A crucial step in hormonal action is the interaction of hormones with receptors on the plasma membrane surface of target cells. The formation of hormone-receptor complexes allows the transduction of extracellular signals into the cell to elicit a variety of biological responses.

[0065] Polypeptides described herein can be screened for their antagonistic or inverse agonistic properties using the cAMP accumulation assay. In one such assay, cells expressing PTH-1 receptor on the cell surface are incubated with native PTH(1-84) for 5-60 minutes at 37°C, in the presence of 2 mM IBMX (3-isobutyl-1-methyl-xanthine, Sigma, St. Louis, MO). Cyclic AMP accumulation is measured by specific radio-immunoassay. A compound that competes with native PTH(1-84) or PTH(1-34), or any fragments thereof, for binding to the PTH-1 receptor, and that inhibits the effect of native PTH(1-84)

or PTH(1-34) on cAMP accumulation, is considered a competitive antagonist. Such a compound would be useful for treating hypercalcemia.

[0066] Conversely, a PTH analog described herein or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, but which still prevents native PTH(1-84) or PTH(1-34) activation of cAMP accumulation (presumably by blocking the receptor activation site) is considered a non-competitive antagonist. Such a compound would also be useful for treating hypercalcemia.

Therapeutic Uses of PTH Analogs

[0067] Some forms of hypercalcemia are related to the interaction between PTH and PTHrP and the PTH-1 and PTH-2 receptors. Hypercalcemia is a condition in which there is an abnormal elevation in serum calcium level; it is often associated with other diseases, including hyperparathyroidism, osteoporosis, carcinomas of the breast, lung and prostate, epidermoid cancers of the head and neck and of the esophagus, multiple myeloma, and hypernephroma.

[0068] By "agonist" is intended a ligand capable of enhancing or potentiating a cellular response mediated by the PTH-1 receptor. By "antagonist" is intended a ligand capable of inhibiting a cellular response mediated by the PTH-1 receptor. Whether any peptide of the present invention is classified as an "agonist" or "antagonist," *i.e.,* a compound that can enhance or inhibit such a cellular response, can be determined using art-known protein ligand/receptor cellular response or binding assays, including those described elsewhere in this application.

[0069] In accordance with yet a further aspect of the invention, there is provided a method for treating a medical disorder that results from altered or excessive action of the PTH-1 receptor, comprising administering to a patient therapeutically effective amount of a compound of the invention or a derivative thereof sufficient to inhibit activation of the PTH-1 receptor of said patient.

[0070] In this embodiment, a patient who is suspected of having a disorder resulting from altered action of the PTH-1 receptor can be treated using compounds of the invention or derivatives thereof of the invention which are a selective antagonists of the PTH-1 receptor. Such antagonists include compounds of the invention or derivatives thereof of the invention which have been determined (by the assays described herein) to interfere with PTH-1 receptor-mediated cell activation or other derivatives having similar properties.

[0071] To administer the antagonist, the appropriate compound of the invention or a derivative thereof is used in the manufacture of a medicament, generally by being formulated in an appropriate carrier or excipient such as, *e.g.*, physiological saline, and administered intravenously, intramuscularly, subcutaneously, orally, or intranasally, at a dosage that provides adequate inhibition of a compound of the invention or a derivative thereof binding to the PTH-1 receptor. Typical dosage would be 1 ng to 10 mg of the peptide per kg body weight per day.

[0072] It will be appreciated to those skilled in the art that the invention can be performed within a wide range of equivalent parameters of composition, concentration, modes of administration, and conditions without departing from the spirit or scope of the invention or any embodiment thereof.

[0073] Having now fully described the invention, the same will be more readily understood by reference to specific examples which are provided by way of illustration, and are not intended to be limiting of the invention, unless herein specified.

EXAMPLES

[0074] The following protocols and experimental details are referenced in the examples that follow.

[0075] *Peptides.* Peptides are prepared on automated peptide synthesizers (model 430A PE, Applied Biosystems, Foster City, CA, or Model 396 MBS Advanced Chem Tect, Louisville, KY) using FMOC main-chain protecting group chemistry,

HBTU/HOBt/DIEA (1:1:2 molar ratio) for coupling reactions, and TFA-mediated cleavage/sidechain-deprotection (MGH Biopolymer Synthesis Facility, Boston, MA). All peptides are desalted by adsorption on a C18-containing cartridge, and purified further by HPLC. The dry peptide powders are reconstituted in 10 mM acetic acid and stored at − 80 °C. The purity, identity, and stock concentration for each peptide is secured by analytical HPLC, Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and amino acid analysis. Radiolabeling is performed using ^{125}I-Na (2,200 Ci/mmol, NEN) and chloramine-T; the resultant radioligands are purified by HPLC.

[0076] *Cell Culture.* The cell line HKRK-B28 (Shimizu, *et al., Biochem. 41*:13224-33 (2002)) was derived from the porcine kidney cell line, LLC-PK_1 by stable transfection with plasmid DNA encoding the opossum/rat hybrid P1R and expresses ~280,000 receptors per cell. The HKRK-B7 cell line was derived from the LLC-PK_1 cell line by stable transfection with DNA encoding human P1R. HKRK-B7 cells expresses approximately 950,000 human P1R per cell (Takasu, H., *et al., J. Bone Miner. Res. 14*:11-20 (1999)). These cells, as well as COS-7 cells and SaOS-2-B10 cells, are cultured at 27°C in T-75 flasks (75 mm^2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin G (20 units/ml), streptomycin sulfate (20 μg/ml) and amphotericin B (0.05 μg/ml) in a humidified atmosphere containing 5% CO_2. Stock solutions of EGTA/trypsin and antibiotics are from GIBCO; fetal bovine serum is from Hyclone Laboratories (Logan, UT). COS-7 cells sub-cultured in 24-well plates are transfected with plasmid DNA (200 ng per well) encoding the wild-type human P1R or truncated human P1R deleted for residues (24-181) (Shimizu, M., *et al., J. Biol. Chem. 275*:21836-21843 (2000)) that is purified by cesium chloride/ethidium bromide density gradient centrifugation, and FuGENE 6 transfection reagent (Roche Indianapolis IN) according to the manufacturer's recommended procedure. All cells, in 24-well plates, are treated with fresh media and shifted to 33 °C for 12 to 24 h prior to assay.

[0077] *cAMP Stimulation.* Stimulation of cells with peptide analogs is performed

in 24-well plates. Cells are rinsed with 0.5 mL of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.5 with HCl) and treated with 200 μL of cAMP assay buffer (Delbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/mL bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 μL of binding buffer containing varying amounts of peptide analog (final volume = 300μL). The medium is removed after incubation for 30 to 60 minutes at room temperature, and the cells are frozen on dry ice, lysed with 0.5 mL 50 mM HCl, and refrozen (~80°C). The cAMP content of the diluted lysate is determined by radioimmunoassay. The EC_{50} response values are calculated using nonlinear regression (see below).

[0078] *Competition Binding.* Binding reactions are performed with HKRK-B28 cells, HKRK-B7 or in COS-7 cells in 24-well plates. The cells are rinsed with 0.5 mL of binding buffer, and then treated successively with 100 μL binding buffer, 100 μL of binding buffer containing various amounts of unlabeled competitor ligand, and 100 μL of binding buffer containing ca. 100,000 cpm of ^{125}I-[M]PTH(1-21), ^{125}I-[Aib^{1,3},Nle^{8},Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]PTH(1-15)NH_2 or ^{125}I-[Aib^{1,3},M]PTH(1-21) (ca. 26 fmol; final volume = 300 μL). Incubations are 4 to 6 h at 4°C, at which time near equilibrium conditions were attained. Cells are then placed on ice, the binding medium was removed, and the monolayer is rinsed three times with 0.5 mL of cold binding buffer. The cells were subsequently lysed with 0.5 mL 5N NaOH and counted for radioactivity. For each tracer and in each experiment, the non-specific binding is determined as the radioactivity that bound in the presence of the same unlabeled peptide at a concentration of 1 μM, and was ~1% of total radioactivity added for each tracer. The maximum specific binding (B_0) is the total radioactivity bound in the absence of competing ligand, corrected for nonspecific binding. Nonlinear regression is used to calculate binding IC_{50} values (see below). Scatchard transformations of homologous competition binding data derived from studies with 26 fmol of ^{125}I-[Aib^{1,3},M]PTH(1-21) are employed for estimations of apparent equilibrium

dissociation constant (k_{Dapp}s) and total number of ligand binding sites (B_{max}), assuming a single class of binding sites and equal affinities of the iodinated and non iodinated ligand.

[0079] *Stimulation of Inositol Phosphate Production.* COS-7 cells transfected as above with P1R-WT are treated with serum-free, inositol-free DMEM containing 0.1% bovine serum albumin and [^{3}H]myo-inositol (NEN, Boston, MA) (2 μCi/mL) for 16 h prior to assay. At the time of the assay, the cells are rinsed with binding buffer containing LiCl (30 mM) and treated with the same buffer with or without a PTH analog. The cells are then incubated at 37 °C for 40 min, after which the buffer was removed and replaced by 0.5 mL of ice cold 5% trichloroacetic acid solution. After 3 h on ice, the lysate is collected and extracted twice with ethyl ether. The lysate is then applied to an ion exchange column (0.5 mL resin bed) and the total inositol phosphates are eluted as described previously (Berridge, M.J., *et al., Biochem. J. 212*:473-482 (1983)), and counted in liquid scintillation cocktail.

Example 1: Results

[0080] Binding assays of PTH derivatives were performed in HKRK-B28 cells. Derivatives of the parent peptide, [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ ID NO: 14), were substituted at positions 1, 2 and/or 3, as indicated. Binding assays (4h at 15°C) were performed with ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]PTH(1-15)NH_2. The results indicate that the parent peptide bound with the highest affinity of the derivatives tested (FIG. 2 and FIG. 3 (A)).

[0081] The parent peptide, [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2, and derivatives thereof substituted at positions 1, 2 and/or 3, as indicated, were assayed for cAMP agonist responses in HKRK-B28 cells (FIG. 3 (C) and FIG. 4). The assays were performed at RT for 30 min. The parent peptide functions as a fully potent and efficacious agonist, the $Deg^{1,3}$-substituted analog is a partial

agonist, and the Bpa^2-substituted analogs lack agonist activity. These results indicate a crucial role for position 2 in stimulating cAMP production.

[0082] The analogs also behave as inverse agonists in COS-7 cells expressing the constitutively active mutant human P1Rs derived from patients with Jansen's disease, P1R-H223R and P1R-H223R/T410P (FIG. 7A and C). The analog [desNH$_2$-Ac$_5$c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH$_2$ behaved as an inverse agonist in COS-7 cells expressing the constitutively active mutant receptor P1R-T410P (FIG. 7B). None of the analogs is an inverse agonist in COS-7 cells expressing the constitutively active mutant receptor P1R-I458R (FIG. 7 D), which appears to be a "locked-on" P1R. These results demonstrate the P1R-selectivity of the new analogs.

[0083] In COS-7 cells expressing P1R-delNt, [Deg^{1,3},BPA^2,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}]PTH(1-21)NH$_2$ strongly inhibited both [Aib^{1,3},Tyr^{34}]hPTH(1-34)NH$_2$ and Ac$_5$c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH$_2$, whereas [Ile^5,Trp^{23},Tyr^{36}]PTHrP(5-36)NH$_2$ was inert on this receptor construct for either agonist ligand (FIG. 8B). These results demonstrate that [Deg^{1,3},BPA^2,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}]PTH(1-21)NH$_2$ is a potent J-domain selective antagonist. The differences in the antagonist actions of [Deg^{1,3},BPA^2,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}]PTH(1-21)NH$_2$ and [Ile^5,Trp^{23},Tyr^{36}]PTHrP(5-36)NH$_2$ on the intact human P1R (Fig 6A) was not due to differences in binding affinities for this receptor, as shown by the similar IC_{50} values observed for these analogs in competition studies performed in HKRK-B7 cells, which stably express the wild-type human P1R (FIG. 9). These results further illustrate that [Deg^{1,3},BPA^2,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}] PTH(1-21)NH$_2$ utilizes a novel mechanism to achieve antagonism, which is different from that used by the conventional N-terminally truncated antagonist, [Ile^5,Trp^{23},Tyr^{36}]PTHrP(5-36)NH$_2$.

[0084] The pharmacologic mechanism by which [Deg^{1,3},BPA^2,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}]PTH(1-21)NH$_2$ and

[$Deg^{1,3}$,Bpa^2,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 function as antagonists was investigated in Figure 10. In this study, the effects of the antagonists at 10 mM, on the dose-response curve elicited by the agonist [$Aib^{1,3}$,Tyr^{34}]hPTH(1-34)NH_2 were examined in HKRK-B7 cells. The presence of either antagonist resulted in a rightward and parallel shift in the agonist response curve, and although true asymptotes were not attained in the curves, the results are fully consistent with a competitive, versus a non-competitive, mechanism of inhibition.

[0085] "N" versus "J" domain selectivity of PIR antagonists was investigated in COS-7 Cells (FIG. 8). cAMP antagonism assays were performed in COS-7 cells transfect with the wild-type P1R (A), or a P1R derivative (P1R-delNt) having most (residues 24-181) of the P1R N domain deleted (B). Cells were treated with the agonist [$Aib^{1,3}$,Tyr^{34}]hPTH(1-34)NH_2 ([$Aib^{1,3}$]PTH(1-34)), which utilizes both N and J domains for affinity/potency, or with [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 ([$Ac5c^1$]PTH(1-14)), which uses only the J domain for affinity/potency, at the concentrations indicated in the key, so as to elicit half-maximum cAMP responses in the absence of antagonist (none). The analogs PTHrP(5-36) and $Deg^{1,3}$,Bpa^2-PTH(1-21) were added at 1x10-5 M, as indicated. On the WT receptor, PTHrP(5-36) antagonizes PTH(1-34) analog more effectively than does $Deg^{1,3}$,Bpa^2-PTH(1-21), but the PTH(1-21) analog antagonizes PTH(1-14), more effectively than does PTHrP(5-36). On P1R-delNt, $Deg^{1,3}$,Bpa^2-PTH(1-21) antagonizes either agonist, whereas PTHrP(5-36) lacks antagonist capability. Thus, PTHrP(5-36) is an N domain-selective antagonist, whereas $Deg^{1,3}$,Bpa^2-PTH(1-21) is a J domain-selective antagonist. The analog $Deg^{1,3}$,Bpa^2-PTH(1-14) behaved similarly in these assays to $Deg^{1,3}$,Bpa^2-PTH(1-21).

[0086] Direct structural analyses of these analogs, as free peptides, or potentially in complex with the PTH-1 receptor, could provide valuable insights into the ligand structures that allow a ligand to act as an agonist on the PTH-1 receptor. In this regard, the information derived from the data set described herein could be of use in the design of peptide mimetics for the PTH-1 receptor. Approaching

this problem from the standpoint of the native PTH peptide sequence is made difficult by the conformational diversity that is possible at each position in the peptide backbone chain. The incorporation of stereochemically constrained amino acids, such as Aib, into the peptide chain, lessens this problem, as it serves to nucleate predictable peptide structures. Thus, the approach can facilitate the *de novo* design of peptide or nonpeptide agonists for the PTH-1 receptor. Given the recently proven utility of PTH(1-34) in treating osteoporosis (Neer, R.M., *et al., N.E.J.M. 344*:1434-1441 (2001)), such agonists should have important medical impact.

[0087] Computer models of the interaction with native PTH are being developed (Jin, L., *et al., J. Biol. Chem. 275*:27238-27244 (2000); Rölz, C., and Mierke, D.F., Biophysical Chemistry (2000)). The above described experiments with the truncated PTH-1 receptor, P1R-delNt, provide some insights into interactions betwen PTH analogs and the PTH receptors, as they demonstrate that the enhancing effects of the Aib substitutions at positions 1 and 3 are mediated through the juxtamembrane region (J domain) of the receptor containing the extracellular loops and transmembrane domains. This finding is consistent with the cumulative crosslinking and mutational data on the PTH/PTH-1 receptor interaction, which indicate that residues in the (1-14) domain of PTH interact primarily, if not exclusively, with the receptor's J domain, as opposed to its amino-terminal extracellular domain (N domain) (Bergwitz, C., *et al., J. Biol. Chem. 271*:26469-26472 (1996); Hoare, S.R.J., *et al., J. Biol. Chem 276*:7741-7753 (2001); Behar, V., *et al., J. Biol. Chem. 275*:9-17 (1999); Shimizu, M., *et al., J. Biol. Chem. 275*:19456-19460 (2000); Luck, M.D., *et al., Molecular Endocrinology 13*:670-680 (1999); Shimizu, M., *et al., J. Biol. Chem. 275*:21836-21843 (2000); Carter, P.H., and Gardella, T.J., *Biochim. Biophys. Acta 1538*:290-304 (2001); Gardella, T.J., *et al., Endocrinology 132*:2024-2030 (1993); Bisello, A., et al., J. Biol. Chem. 273:22498-22505 (1998)).

[0088] Two modes of antagonism are now recognized at the P1R. N domain inhibition (A) is utilized by most conventional P1R antagonists, such as

PTHrP(5-36) and PTHrP(7-34) analogs, and is based on the derivation of binding energy primarily from interactions between the (21-34) region of the ligand and the P1R N domain. This mechanism is effective for of inhibition of N-domain-dependent agonists, such as PTH(1-34), but not for N domain-independent agonists, such as PTH(1-19). J domain inhibition (B) is utilized by the novel analogs described herein, and is based on the derivation of binding energy primarily or wholly from interactions between the (1-20) region of the ligand and the J domain of the P1R. The results also demonstrate that the antagonizing effects of the position-1, 2 and/or 3 modifications are mediated through the J domain. This mechanism is effective for inhibition of J-domain-dependent agonists, such as PTH(1-14) analogs, but not for N domain-dependent agonists, such as PTH(1-34). A J domain-selective antagonists would be useful for characterizing small-molecules that act as PTH mimetics, since such molecules are likely to bind to the J domain.

[0089] Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that same can be performed by modifying or changing the invention with a wide and equivalent range of conditions, formulations and other parameters thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

[0090] All publications, patents and patent applications mentioned hereinabove are herein incorporated in their entirety and by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A peptide selected from a group consisting of:

(a) $X_{01}X_{02}X_{03}$GluIleGlnLeuX_{04}His$X_{05}X_{06}X_{07}$LysX_{08} (SEQ ID NO: 1),

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; and

(d) N- or C- derivatives thereof;

wherein:

X_{01} and X_{03} are each an α-helix stabilizing residue,

X_{02} is Trp, Bpa, Arg or Val,

X_{04} is is Met or Nle,

X_{05} is Gln, Deg or Asn,

X_{06} is Har or Leu,

X_{07} is α-helix stabilizing residue, Ala or Gly,

X_{08} is an α-helix stabilizing residue, Trp, Tyr or His; and

wherein said peptide binds selectively to the J domain of P1R.

2. The peptide of claim 1, wherein said α-helix stabilizing residue is selected from the group consisting of Ac_5c, Ac_3c, Deg, Aib or the desamino form of Ac_5c, Ac_3c, Deg, or Aib.

3. The peptide of claim 1, wherein said peptide is selected from:

(a) Ac5cBpaAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ ID NO:13);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

4. The peptide of claim 1, wherein said peptide is selected from:

(a) Ac_5cValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 14);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

5. The peptide of claim 1, wherein said peptide is selected from:

(a) desamino Ac_5cValAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 15);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

6. The peptide of claim 1, wherein said peptide is selected from:

(a) desamino AibValAibGluIleGlnLeuMetHisGlnHarAla-LysTrpNH_2 (SEQ ID NO: 16);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

7. The peptide of claim 1, wherein said peptide is selected from:

(a) Ac_5cTrpAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ ID NO: 17);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

8. The peptide of claim 1, wherein said peptide selected from:

(a) $Ac_5cBpaAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2$ (SEQ ID NO: 18),

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

9. The peptide of claim 1, wherein said peptide selected from:

(a) $Ac_5cArgAibGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2$ (SEQ ID NO: 19),

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

10. The peptide of claim 1, wherein said peptide selected from:

(a) $DegValDegGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2$ (SEQ ID NO: 20);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

11. The peptide of claim 1, wherein said peptide selected from:

(a) $DegTrpDegGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2$ (SEQ ID NO: 21);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11,

1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

12. The peptide of claim 1, wherein said peptide selected from:

(a) $DegBpaDegGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2$ (SEQ ID NO: 22);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

13. The peptide of claim 1, wherein said peptide selected from:

(a) $Ac_5cTrpAibGluIleGlnLeuNleHisGlnHarAlaLysTyrNH_2$ (SEQ ID NO: 23);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

14. The peptide of claim 1, wherein said peptide selected from:

(a) $Ac_5cBpaAibGluIleGlnLeuNleHisGlnHarAlaLysTyrNH_2$ (SEQ ID NO: 24);

(b) fragments thereof, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

15. A peptide selected from a group consisting of:

(a) $X_{01}BpaX_{02}GluIleGlnLeuX_{03}HisX_{04}X_{05}X_{06}LysX_{07}LeuAla$

SerValX_{08}ArgX_{09} (SEQ ID NO: 6);

(b) fragments thereof, containing amino acids 1-20, 1-19, 1-18, 1-17, 1-16 or 1-15;

(c) pharmaceutically acceptable salts thereof; and

(d) N- or C- derivatives thereof;

wherein

X_{01} and X_{02} are α-helix stabilizing residues,

X_{02} is Aib, Gln, Deg or Asn,

X_{03} is Met or Nle,

X_{04} is Har or Leu,

X_{05} is an α-helix stabilizing residue, Ala or Gly,

X_{06} is an α-helix stabilizing residue (e.g. Aib) or Lys,

X_{07} is an α-helix stabilizing residue, Trp or His,

X_{08} is Arg or Glu and X_{09} is Tyr or Met; and

wherein said peptide binds selectively to the J domain of P1R.

16. The peptide of claim 15, said peptide selected from:

(a) DegBpaDegGluIleGlnLeuNleHisGlnHarAlaLysTrpLeuAla SerValArgArgTyrNH_2 (SEQ ID NO: 25);

(b) fragments thereof, containing amino acids 1-11, 1-12 or 1-13;

(c) pharmaceutically acceptable salts thereof; or

(d) N- or C- derivatives thereof.

17. The peptide of claim 1 or 15, wherein said peptide is labeled.

18. The peptide of claim 17, wherein said peptide is labeled with a fluorescent label.

19. The peptide of claim 17, wherein said peptide is labeled with a

chemiluminescent label.

20. The peptide of claim 17, wherein said peptide is labeled with a bioluminescent label.

21. The peptide of claim 17, wherein said peptide is labeled with a radioactive label.

22. The peptide of claim 21, wherein said peptide is labeled with ^{125}I.

23. The peptide of claim 21, wherein said peptide is labeled with ^{99m}Tc.

24. A competition binding assay to identify a PTH receptor ligand, which comprises contacting said receptor with a labeled peptide of claim 17 and a candidate receptor ligand, and measuring the label bound to the receptor.

25. A competition binding assay to analyze a PTH receptor ligand, which comprises contacting said receptor, or fragments or derivatives thereof, with a labeled peptide of claim 17 and a candidate receptor ligand, and measuring the label bound to the receptor.

26. A pharmaceutical composition comprising the peptide of claim 1 or 15, and a pharmaceutically acceptable carrier.

27. A method for treating mammalian conditions characterized by increased activity or production of PTH or PTHrP, said method comprising administering to a subject in need thereof an effective inhibitory amount of a peptide of claim 1 or 15.

28. A method for treating mammalian conditions characterized by increased activity or production of PTH or PTHrP, said method comprising administering to a subject in need thereof an effective inhibitory amount of a composition comprising a peptide of claim 1 or 15 and a pharmaceutically acceptable carrier.

29. The method of claim 26 or 27, wherein said condition to be treated is hypercalcemia.

30. The method of claim 28, wherein said condition to be treated is malignant hypercalcemia.

31. The method of claim 26 or 27, wherein said effective amount of said peptide for increasing bone mass is from about 0.01 μg/kg/day to about 1.0 μg/kg/day.

32. The method of claim 26 or 27, wherein the method of administration is parenteral.

33. The method of claim 26 or 27, wherein the method of administration is subcutaneous.

34. The method of claim 26 or 27, wherein the method of administration is nasal insufflation.

35. A method of making the peptide of claim 1 or 15, wherein said peptide is synthesized by solid phase synthesis.

36. The method of making the peptide of claim 1 or 15, wherein said peptide is protected by FMOC.

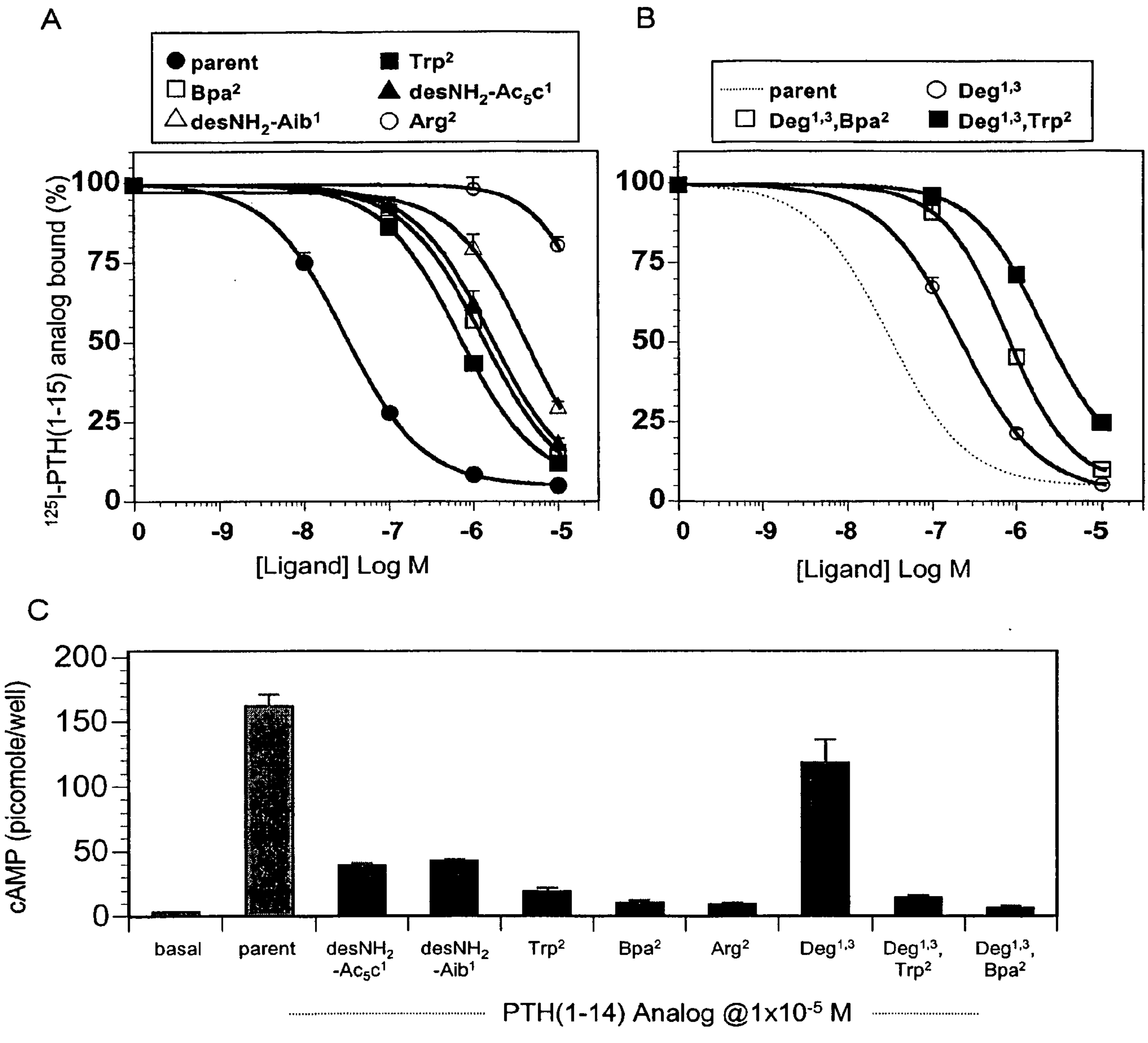
SEQ ID NO.	Peptide	Sequences
	PTH(1-14) peptides	
26	PTH(1-14)NH_2 (native, rat)	Ala-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-NH_2
27	[$Ala,^{3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)NH_2	Ala-Val-Ala-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
14	[Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Ac_5c-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
15	[desNH2-Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	(desNH2)Ac_5c-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
16	[desNH2-Aib^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	(desNH2)Aib-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
17	[Ac_5c^1,Trp^2,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Ac_5c-Trp-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
18	[Ac_5c^1,Bpa^2,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Ac_5c-Bpa-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
19	[Ac_5c^1,Arg^2,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Ac_5c-Arg-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
20	[$Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Deg-Val-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
21	[$Deg^{1,3}$,Trp^2,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Deg-Trp-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
22	[$Deg^{1,3}$,Bpa^2,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2	Deg-Bpa-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH_2
23	[Ac_5c^1,Trp^2,Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},Tyr^{14}]PTH(1-14)NH_2	Ac_5c-Trp-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Tyr-NH_2
24	[Ac_5c^1,Bpa^2,Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},Tyr^{14}]PTH(1-14)NH_2	Ac_5c-Bpa-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Tyr-NH_2
25	[$Deg^{1,3}$,Bpa^2,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}]PTH(1-21)NH_2	Deg-Bpa-Deg-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp- Leu-Ala-Ser-Val-Arg-Arg-Tyr^{*}-NH_2
	N-truncated peptides	
28	[Aib^3,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Arg^{19},Tyr^{21}]PTH(3-21)NH_2	Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Leu-Ala-Ser-Val-Arg-Arg-Tyr-NH_2
29	[Ile^5,Trp^{23},Tyr^{36}]PTHrP(5-36)NH_2	Ile-Gln-Leu-Leu-His-Asp-Lys-Gly-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg- Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr*-NH_2
31	[Ile^5,Leu^{11},D-Trp^{12},Trp^{23},Tyr^{36}]PTHrP(5-36)NH_2	Ile-Gln-Leu-Leu-His-Asp-Leu-DTrp-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr-NH_2
	^{125}I-PTH tracer radioligand	
32	[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14},Tyr^{15}]PTH(1-15)NH_2	Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Tyr*-NH_2

Figure 1

	Peptides		IC50			
	PTH(1-14) peptides				nM	n
##	parent		30	±	7	3
##	desNH$_2$-Aib^1		4,500	±	700	4
##	desNH$_2$-AC$_5$C^1		1,800	±	100	4
##	Arg^2		25,000	±	2,000	4
##	Trp^2		770	±	110	4
##	Bpa^2		1,400	±	200	4
##	Deg^{1,3}		230	±	50	3
##	Deg^{1,3},Trp^2		2,700	±	300	3
##	Deg^{1,3},Bpa^2		840	±	110	3
	Other peptides					
##	rPTH(1-34)		4.8	±	0.8	3
##	PTHrP(5-36)		5.5	±	1.0	3
##	[Aib3,M]PTH(3-21)		750	±	90	3
##	[Aib1,3,M]PTH(1-21)		18	±	4	3

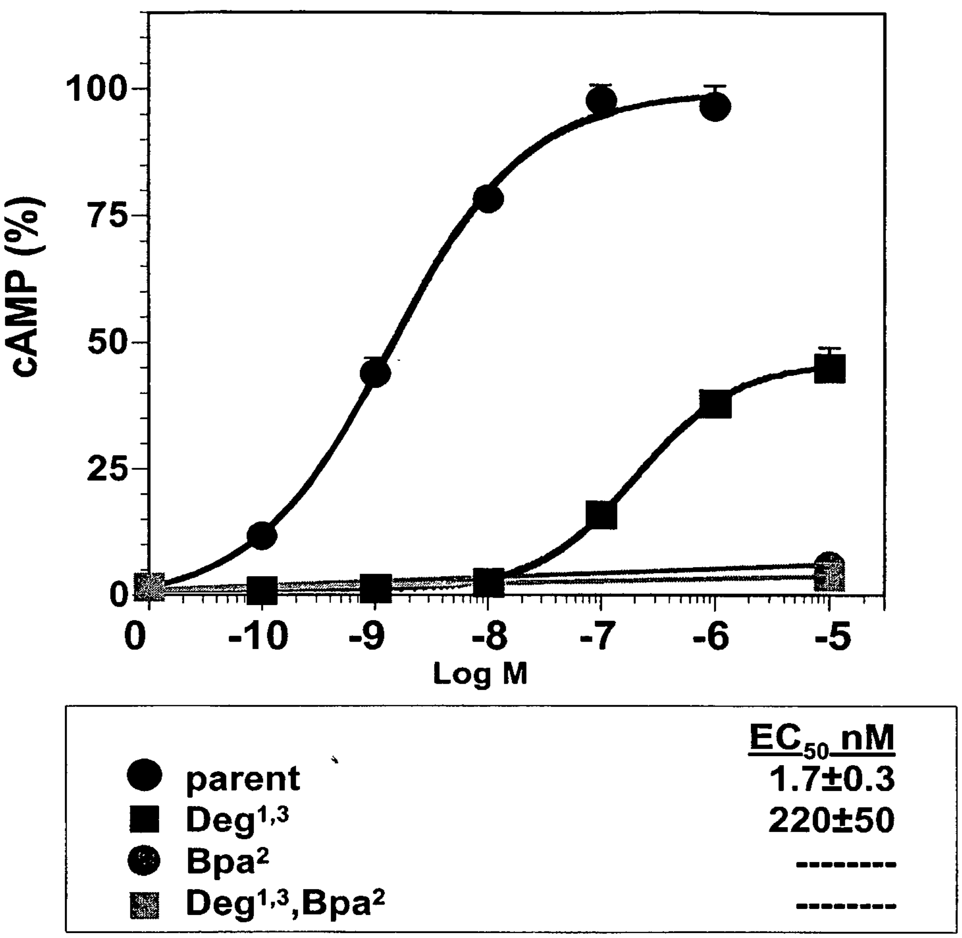
Figure 2

Figure 3



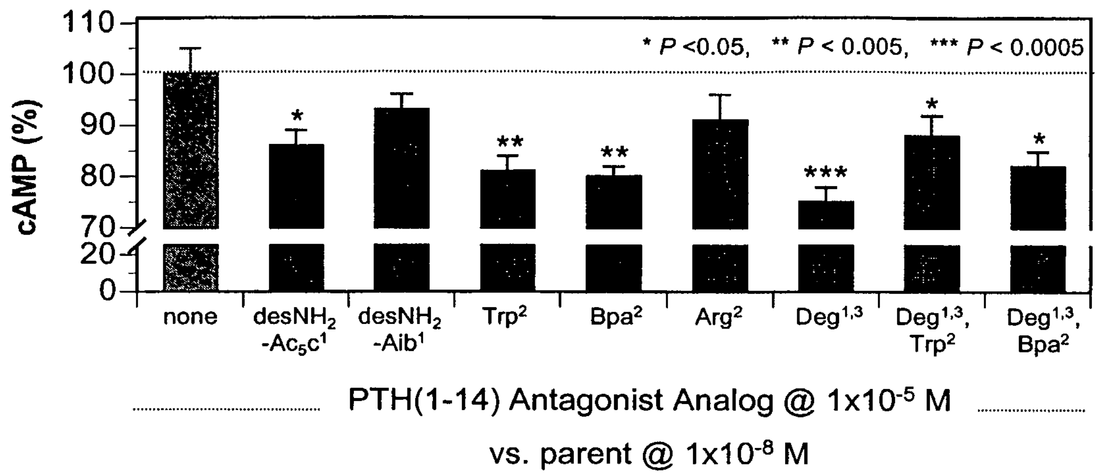
Functional Responses in HKRK-B28 Cells. Binding (A and B) and cAMP agonism/partial agonism assays (C) were performed in HKRK-B28 cells. The parent peptide was [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH2 and derivatives thereof were substituted at positions 1, 2 and/or 3, as indicated. Binding assays (4h @ 15□C) were performed with ^{125}I-[Aib1,3,Nle8,Gln10,Har11,Ala12,Trp14,Tyr15]PTH(1-15)NH2 tracer. cAMP assays were performed at RT for 30 min. Relative to the parent, the substituted analogs lack appreciable agonist activity.

Figure 4



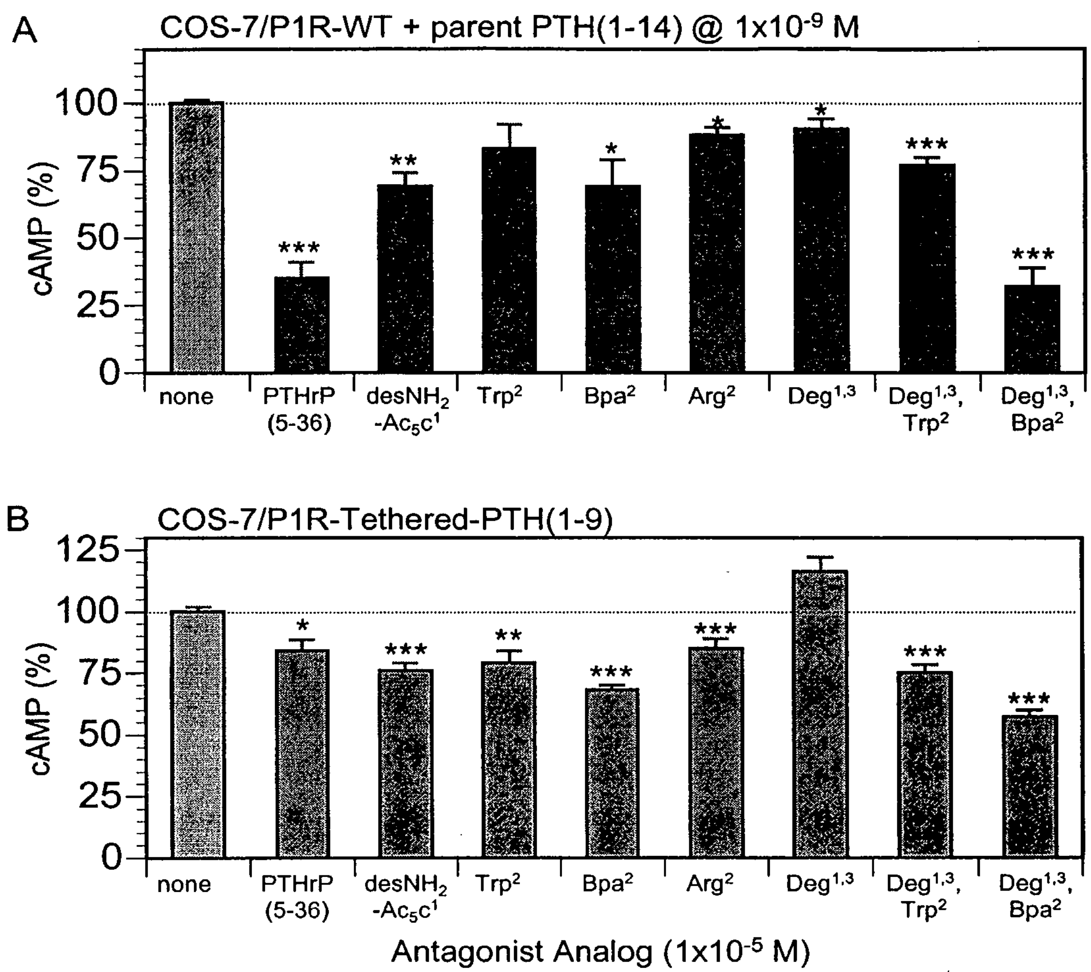
cAMP Responses in HKRK-B28 Cells. The parent peptide, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH2, and derivatives thereof substituted at positions 1, 2 and/or 3, as indicated, were assayed for cAMP agonist responses in HKRK-B28 cells. The parent peptide functions as a fully potent and efficacious agonist, the Deg1,3-substituted analog is a partial agonist, and the Bpa2-substituted analogs lack agonist activity.

Figure 5



Antagonism Assays in HK-RK-B28 Cells. cAMP antagonism assays were performed in HKRK-B28 cells. Cells were treated with the J domain-selective agonist, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH2 (parent) at 10 nM, either alone (none) or with a candidate antagonist peptide (10 μM), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

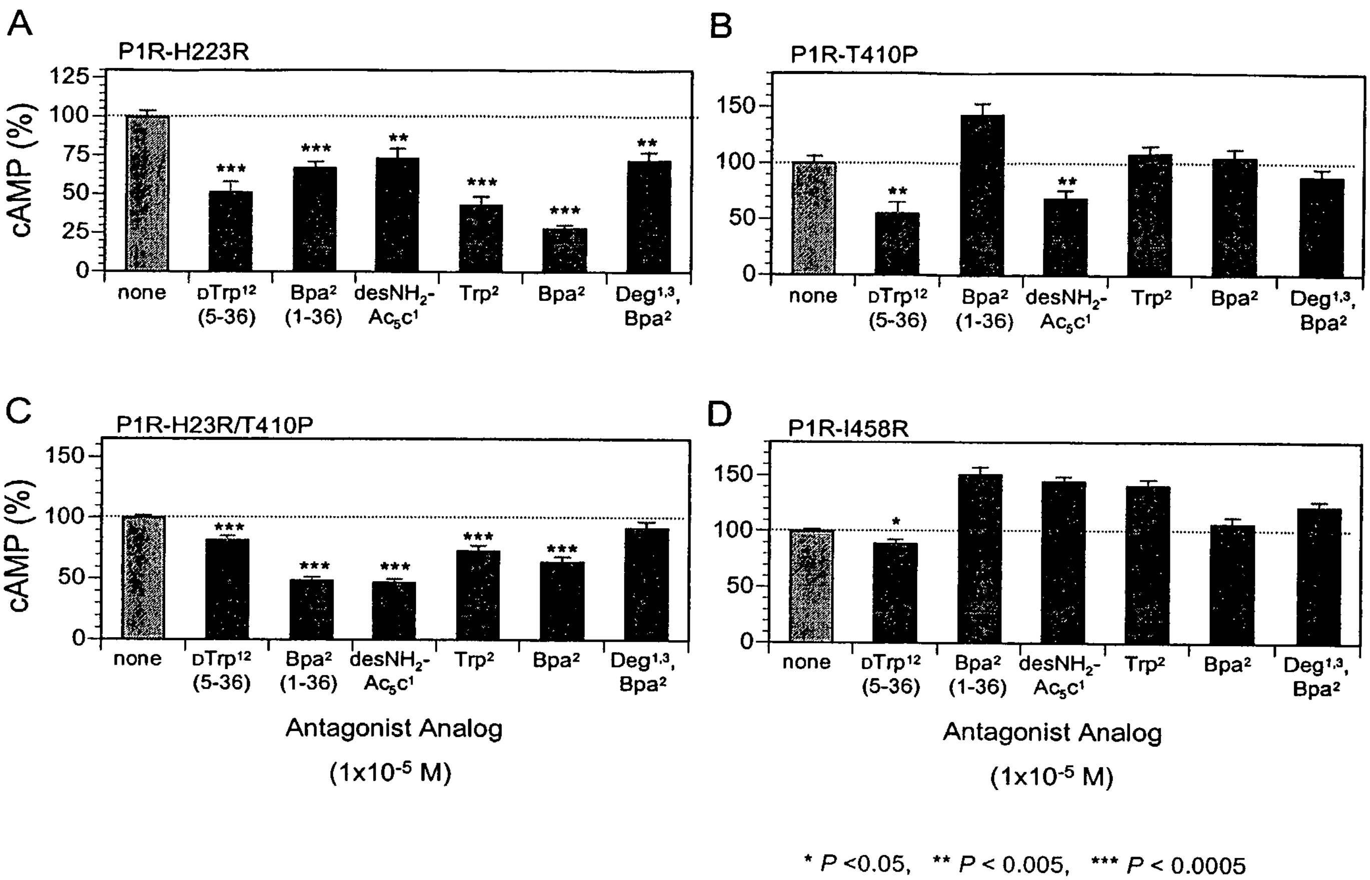
Figure 6



* P <0.05, ** P < 0.005, *** P < 0.0005

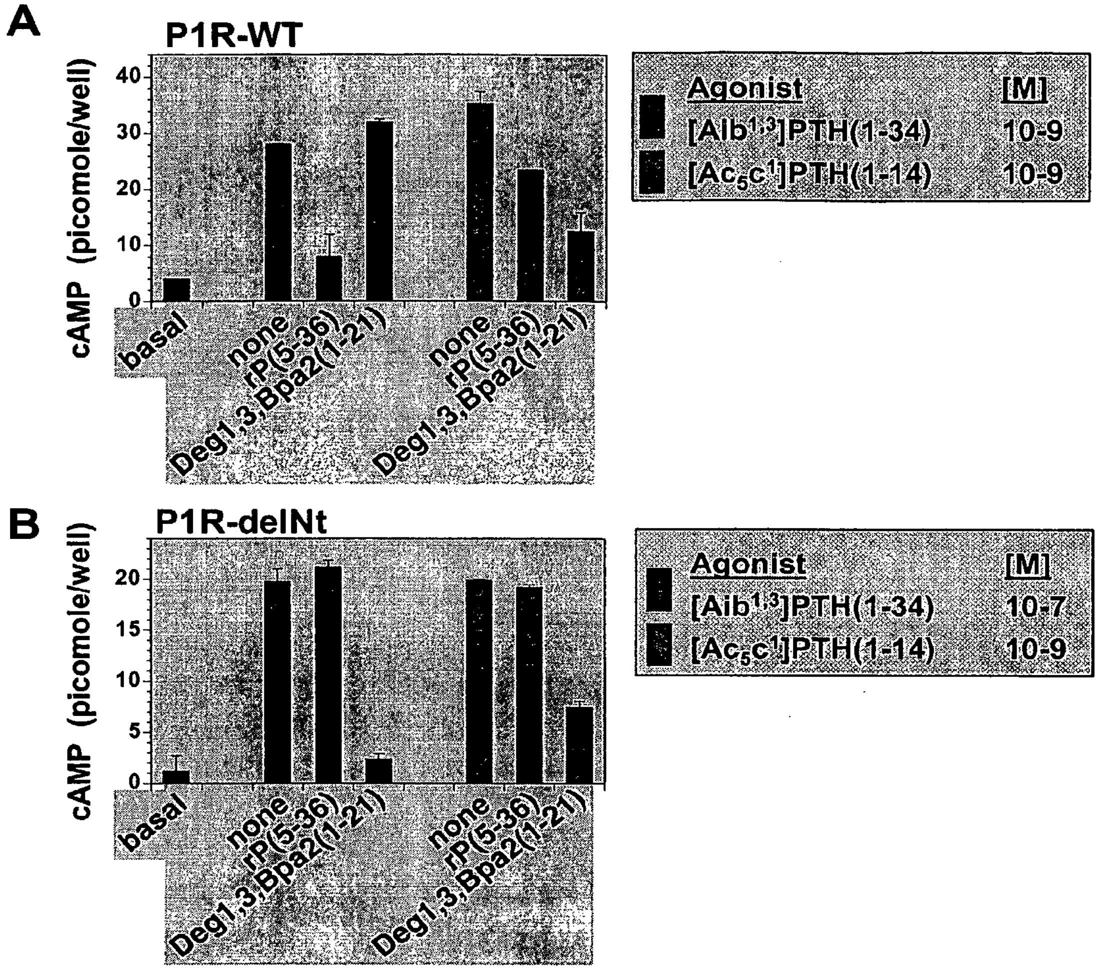
Antagonism Assays in COS-7 Cells. cAMP antagonism assays were performed in COS-7 cells transfect with the wild-type P1R (A), or a constitutively active P1R derivative having the first 9 residues of PTH tethered to TM1 of the P1R and in place of the P1R N-terminal domain (inset), B). In A, cells were treated with the J domain-selective agonist, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH2 (parent) at 1 nM, alone (none) or with a candidate antagonist peptide (10 μM), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated, or [I5,W23,Y36]PTHrP(5-36) analog. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

Figure 7



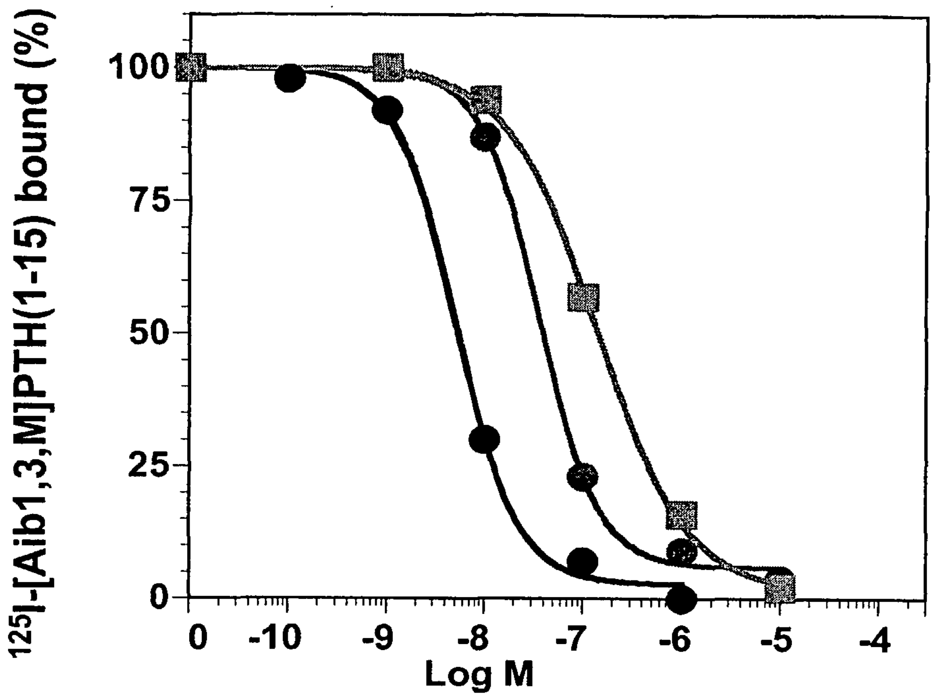
Inverse Agonist Responses in COS-7 Cells. COS-7 cells were transfect with the constitutively active P1Rs: P1R-H223R (A), P1R-T410P (B), P1R-H223R/T410P (C), or P1R-I458R (D) and then were incubated (30 min@R.T.) either in the absence of peptide (none) or in the presence of the indicated antagonist/inverse agonist peptide (10 μM), and cAMP was measured by RIA. Asterisks indicate significant reductions in cAMP levels, compared to untreated cells (none).

Figure 8



"N" versus "J" Domain selectivity of PIR Antagonists in COS-7 Cells. cAMP antagonism assays were performed in COS-7 cells transfect with the wild-type P1R (A), or a P1R derivative (P1R-delNt) having most (residues 24-181) of the P1R N domain deleted (B). Cells were treated with the agonist [Aib1,3,Tyr34]hPTH(1-34)NH2 ([Aib1,3]PTH(1-34)), which utilizes both N and J domains for affinity/potency, or with [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH2 ([Ac5c1]PTH(1-14)), which uses only the J domain for affinity/potency, at the concentrations indicated in the key, so as to elicit half-maximum cAMP responses in the absence of antagonist (none). The analogs PTHrP(5-36) and Deg1,3,Bpa2-PTH(1-21) were added at 1x10-5 M, as indicated. On the WT receptor, PTHrP(5-36) antagonizes PTH(1-34) analog more effectively than does Deg1,3,Bpa2-PTH(1-21), but the PTH(1-21) analog antagonizes PTH(1-14), more effectively than does PTHrP(5-36). On P1R-delNt, Deg1,3,Bpa2-PTH(1-21) antagonizes either agonist, whereas PTHrP(5-36) lacks antagonist capability. Thus, PTHrP(5-36) is an N domain-selective antagonist, whereas Deg1,3,Bpa2-PTH(1-21) is a J domain-selective antagonist. The analog Deg1,3,Bpa2-PTH(1-14) behaved similarly in these assays to Deg1,3,Bpa2-PTH(1-21).

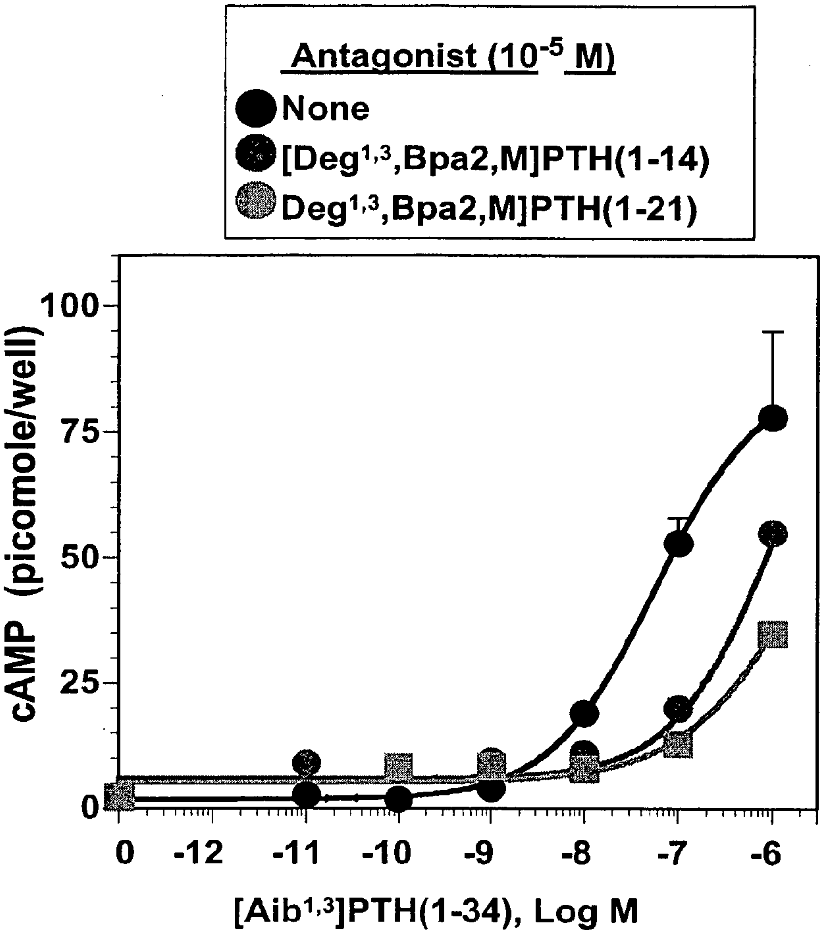
Figure 9



	IC_{50}(nM)
● PTH(1-34)	2
● PTHrP(5-36)	50
■ [Deg^{1,3},Bpa^{2},Y^{15},M]PTH(1-21)	150

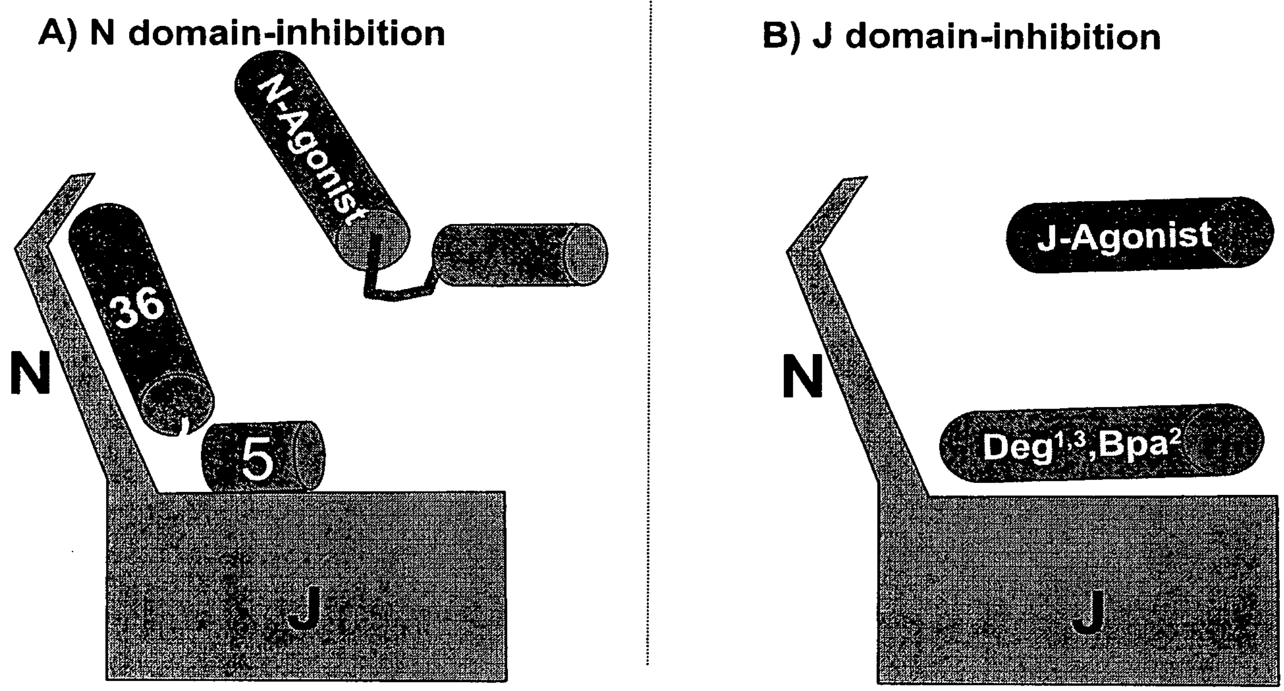
Competition Binding Assays in HKRK-B7 Cells. Binding assays were performed in HKRK-B7 cells, which express the wild-type hP1R, using ^{125}I-[Aib1,3,Nle8,Gln10,Har11,Ala12,Trp14,Tyr15]PTH(1-15)NH2 as a tracer radioligand and the indicated unlabeled peptides as competitors. PTH(1-34) is [Tyr34]hPTH(1-34)NH2.

Figure 10



Competitive Antagonism on P1R-delNt. COS-7 cells transfected with P1R-delNt were stimulated with varying concentrations of the agonist [Aib1,3,Tyr34]hPTH(1-34)NH2 ([Aib1,3]PTH(1-34)), either in the absence of antagonist (green circles) or in the presence of an antagonist, [Deg1,3,Bpa2,M]PTH(1-14) (red circles) or [Deg1,3,Bpa2,M]PTH(1-21) (yellow squares) each at 1x10-5 M, as indicated in the figure key. Each antagonist causes a parallel, right-ward shift in the agonist dose-response curve , which is consistent with a competitive mechanism of inhibition.

Figure 11



Two Modes of Competitive Inhibition at the P1R. Two modes of antagonism are now recognized at the P1R. N domain inhibition (A) is utilized by most conventional P1R antagonists, such as PTHrP(5-36) and PTHrP(7-34) analogs, and is based on the derivation of binding energy primarily from interactions between the (21-34) region of the ligand and the P1R N domain. This mechanism is effective for of inhibition of N-domain-dependent agonists, such as PTH(1-34), but not for N domain-independent agonists, such as PTH(1-14). J domain inhibition (B) is utilized by the novel analogs described herein, and is based on the derivation of binding energy primarily or wholly from interactions between the (1-20) region of the ligand and the J domain of the P1R. This mechanism is effective for inhibition of J-domain-dependent agonists, such as PTH(1-14) analogs, but not for N domain-dependent agonists, such as PTH(1-34). A J domain-selective antagonists would be useful for characterizing small-molecules that act as PTH mimetics, since such molecules are likely to bind to the J domain.